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**14. ABSTRACT:**
   CXCR4 is highly expressed in breast carcinoma cells and is essential for breast cancer metastasis to the lung. CXCR4 is the receptor for CXCL12, a chemokine that is enriched in organs that are targeted by metastatic breast cancer, such as lung and liver. The molecular mechanisms of CXCR4-mediated breast cancer metastasis however are poorly understood. In this project we test the hypothesis that Rac proteins are essential for CXCR4-mediated breast carcinoma cell proliferation and survival, thereby contributing to breast cancer metastasis. The Rac proteins examined comprise Rac1, Rac1b and Rac3. In Task 1, we investigate the role of Rac proteins in CXCL12-regulated breast carcinoma cell proliferation and survival *in vitro*. In Task 2, we will determine the contribution of these Rac proteins to breast cancer metastasis *in vivo*. These approaches should allow us to validate Rac-controlled signaling proteins as novel therapeutic targets for metastatic breast cancer. The research performed in this first year of funding largely pertains to Task 1. We showed that CXCL12 stimulates Rac1 activity in breast carcinoma cells, suggesting that Rac1 is likely to play a role in CXCL12- mediated functions in these cells. We also discuss efforts to specifically downregulate the Rac1b splice form.

**15. SUBJECT TERMS**
cancer cell biology, metastasis models, chemokines, Rac1, Rac1b, Rac3, CXCL12, CXCR4, proliferaton, survival, RNA interference

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INTRODUCTION

This project examines the roles of the small GTPases Rac1 and Rac3 in CXCR4-mediated metastasis of breast carcinoma cells. CXCR4 is highly expressed in breast carcinoma cells and is the receptor for CXCL12, a chemokine that is produced in abundance in organs that are targeted by metastatic breast cancer, such as lung and liver. Rac1b is a splice form of Rac1 that is constitutively active and expressed in breast cancer tissue [1] and therefore the specific role of this splice form in CXCR4-mediated breast carcinoma metastasis will be examined separate from the role of Rac1 itself. The first aim is to determine the specific roles of Rac1, Rac1b and Rac3 in CXCL12-stimulated carcinoma cell survival and proliferation. The second aim is to examine the roles of Rac1, Rac1b and Rac3 in two animal models of breast cancer metastasis, the first using orthotopic mammary fat pad-implanted human breast carcinoma cells (spontaneous metastasis) and the second tail vein-injected cells (experimental metastasis). These approaches should allow us to validate the potential of Rac proteins and signaling elements controlled by these proteins as novel drug targets for metastatic breast cancer.

Note:
Tasks 1 and 2, as outlined in the Body of the report below, refer to the Tasks described in the revised Statement of Work, effective June 8, 2006.

BODY

**Task 1. To determine the role of Rac proteins in CXCL12-regulated functions in breast carcinoma cells *in vitro*.**

Task 1a: Determine the contribution of Rac1 and Rac3 to CXCL12-stimulated proliferation and survival of breast carcinoma cells.

Choice of cell lines. To ensure that our results can be generalized, we will use two breast carcinoma cell lines. In our application we proposed to employ the DU4475 and MDA-MB-231 cell lines. DU4475 cells express a very high level of CXCR4 mRNA [2] and protein (our unpublished data). MDA-MB-231 cells express a 5-fold lower level of CXCR4 mRNA and protein, but their metastatic potential has been extensively characterized [2;3].

Although the DU4475 cell line was derived from a metastatic nodule from a patient with advanced breast cancer, recent observations have shown that these cells are not metastatic when injected orthotopically in the mouse mammary fat pad (H. Nakshatri, U. Indiana, personal communication), makes them inappropriate for our studies.

Surprisingly, MDA-MB-231 cells that we obtained from ATCC showed negligible expression levels of CXCR4, whereas DU4475 cells displayed robust expression (Fig. 1). We found similar low levels of CXCR4 in cells that we obtained from two colleagues (data not shown). Interestingly, work from the group of H. Nakshatri had shown that MDA-MB-231 cells tend to rapidly lose cell surface expression of CXCR4 when cultured in vitro, but regain expression after implantation in the mouse mammary fat pad [4]. Thus, we contacted Dr. Nakshatri to request a vial of mammary fat pad-isolated MDA-MB-231 (termed T231). After a delay of several months we obtained these cells from Dr. Nakshatri. The considerable lag time was due to the fact that Dr. Nakshatri had to reisolate fresh cells after orthotopic transplantation. Determination of the CXCR4 expression levels showed that the T231 that we obtained from Dr. Nakshatri indeed displayed significantly higher expression levels than the other cultures of MDA-MB-231 that we have tested (Fig. 1).

CXCL12 significantly stimulates the proliferative behavior of T231 cells, both in the presence of full serum and low serum (1%) conditions (Fig. 2). We are currently examining whether short interfering RNA- (siRNA)-mediated depletion of Rac1 or Rac3 inhibits CXCL12-stimulated proliferation of T231 cells. A technical problem that we have experienced with these functional studies is the variability in the quality of the CXCL12 preparations that we have obtained from Peprotech, a company with a very good reputation in the chemokine field. We recently have purchased CXCL12 from R&D and are currently testing this preparation.
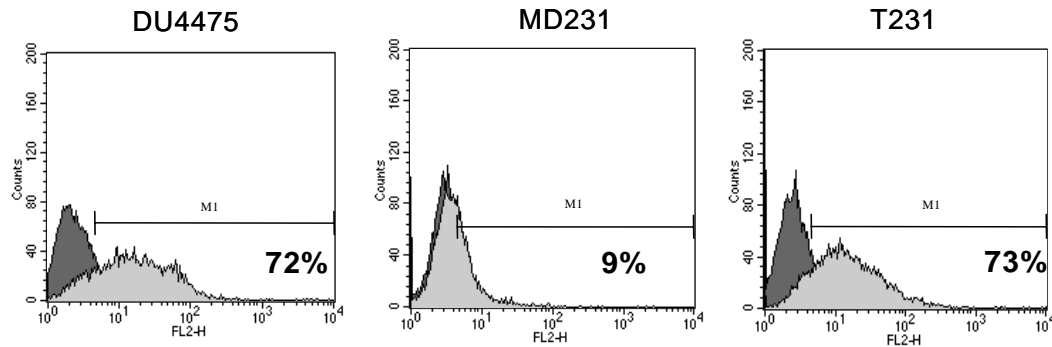
**Figure 1: Flow cytometric analysis of CXCR4 surface expression in human breast carcinoma cell lines**. Shown are the isotype control (dark gray area) and CXCR4-PE (light gray area) populations. Percentage of cells above isotype control background are indicated. Results shown are representative of 4 independent experiments.
**Methods**: Cells were either counted ($5 \times 10^5$ cells/condition) and spun down (DU4475 suspension) or trypsinized (trypsin neutralized with trypsin inhibitor), counted ($5 \times 10^5$ cells/condition), and spun down (MD231, TMD231 adherent). Cells were washed once with FACS buffer (1% BSA in PBS with 0.1% $NaN_3$), stained with either an isotype control antibody (mouse IgG2b-PE, clone 133303, R&D Systems) or CXCR4 antibody (mouse monoclonal anti-human CXCR4-PE, clone 44717, R&D Systems) for 20 min at room temperature, and then washed two times with FACS buffer. The cells were fixed with 1% formaldehyde in PBS and stored at 4°C until analysis (within 1-2 days after staining) on a FACS Calibur (BD Biosciences). Approximately 10,000 events were analyzed for each cell line/antibody.
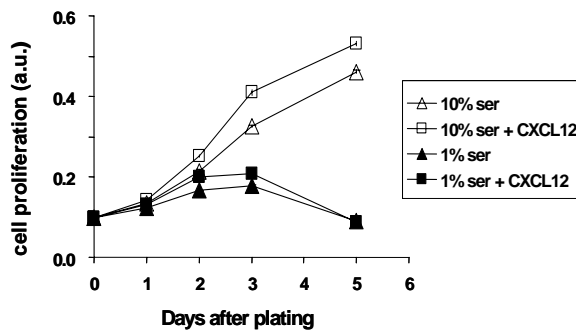


**Figure 2: CXCL12 stimulates the proliferation of T231 cell**s. Cells were plated at a density of $1 \times 10^4$ cells/well in 96-well plates with 6 replicates/condition in the presence of either 10% or 1% FBS and the presence or absence of 100 ng/ml CXCL12 (Peprotech). Cell proliferation was measured using the SRB colorimetric assay (Skehan, P et.al. J.Natl.Cancer Inst., *82*: 1107-1112, 1990). The data shown are the mean of 6 wells (-/+ SEM).

Task 1b: Determine whether Rac1 and Rac3 are activated by CXCL12 in breast carcinoma cell lines.

We examined whether CXCL12 activates Rac1 in breast carcinoma cells using a well-established method that allows the pulling down of activated Rac proteins from cell lysates [5]. We found that CXCL12 indeed enhances Rac1 activation in T231 cells within

then minutes of application of CXCL12 (Fig. 3). We are still optimizing these results, by trying to decrease the background signal in the absence of serum and also plan to perform a detailed kinetic analysis of CXCL12-mediated Rac1 activation.



**Figure 3: CXCL12 stimulates Rac1 activity in T231 cells.** Cells were plated in 10 cm dishes and serum starved overnight. One plate of cells was treated with 100 ng/ml CXCL12 for 5 min, followed by lysis of the cells. 1 mg of cell lysates was used in a Rac pull-down assay to detect the activated form of Rac1 (Pierce). Proteins were separated on a 12% gel, transferred to PVDF membrane, and probed with antibodies to Rac1 (Upstate, mAb clone 23A8; 1:2000) and $\alpha$-tubulin (Sigma, mAb clone DM1A; 1:5000). Results shown are representative of 2 independent experiments.
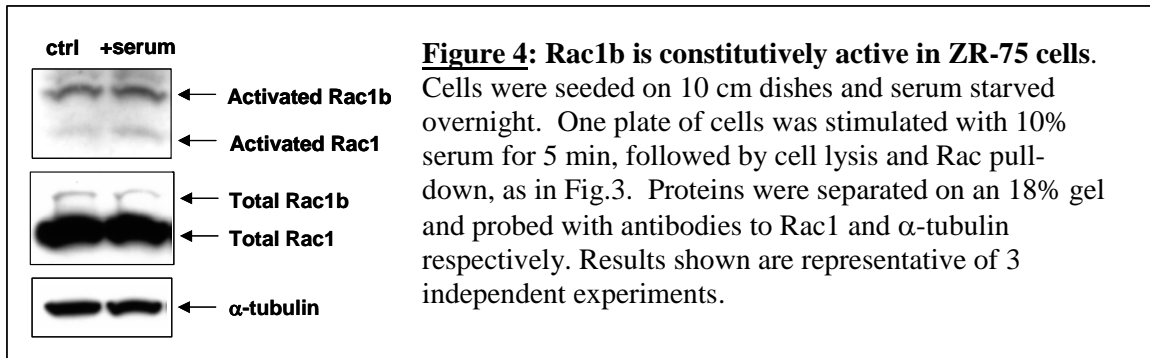
To date, there are no antibodies that specifically recognize Rac3 commercially available (we hitherto have employed quantitative PCR to verify siRNA-mediated knock-down). We therefore decided to generate Rac3-specific monoclonal antibodies in collaboration with Dr. A. Campos (Transduction Labs). Unfortunately, these attempts have not yielded satisfactory antibodies. We recently heard that Dr. A. Cox (University of North Carolina at Chapel Hill) has generated polyclonal antibodies against the unique C-terminal domain of Rac3 and plan to collaborate with her to examine whether CXCL12 also activates endogenous Rac3.
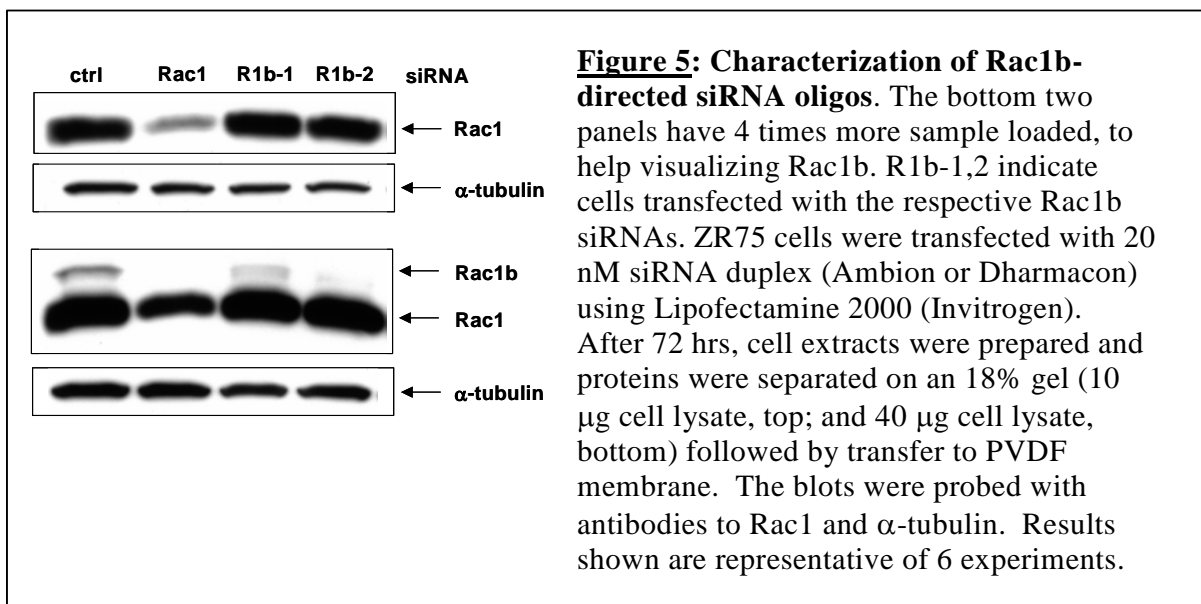
Task 1c: Determine the contribution of Rac1b to CXCL12-stimulated proliferation and survival of breast carcinoma cells.

Rac1b is a splice form of Rac1 that differs from the main splice form of Rac1 by an insert of 19 codons, just C-terminal from the switch II region [1;6;7]. Notably, Rac1b is expressed in breast cancer tissue [1]. Rac1b is constitutively active and does not bind to RhoGDI [1;7;8]. A critical role for Rac1b in breast cancer progression is supported by recent evidence that Rac1b is induced during malignant transformation of mammary epithelial cells and causes oxidative damage to DNA and genomic instability [9].

To evaluate the role of Rac1b in CXCL12-mediated functions in breast carcinoma cells, we first generated and characterized two independent siRNAs targeting Rac1b. We examined the effects of transient transfection of these siRNAs in ZR-75 breast carcinoma cells, since these cells express higher levels of Rac1b than all other breast carcinoma cell lines that we have examined (including MDA-MB-231, MDA-MB-361 and T47D). Although the protein expression level of Rac1b is much lower than that of Rac1 (the major splice form of Rac1) (Fig. 4, middle panel), its level of activation is significantly higher (Fig. 4, top panel). This observation is consistent with the notion that Rac1b is constitutively active [7;8] and underlines the potential importance of Rac1b.

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**Figure 4: Rac1b is constitutively active in ZR-75 cells.** Cells were seeded on 10 cm dishes and serum starved overnight. One plate of cells was stimulated with 10% serum for 5 min, followed by cell lysis and Rac pull-down, as in Fig.3. Proteins were separated on an 18% gel and probed with antibodies to Rac1 and α-tubulin respectively. Results shown are representative of 3 independent experiments.

Transient transfection of either oligo directed against Rac1b caused inhibition of Rac1b expression, albeit to different extents, without affecting the expression of Rac1 (Fig. 5).



**Figure 5: Characterization of Rac1b-directed siRNA oligos.** The bottom two panels have 4 times more sample loaded, to help visualizing Rac1b. R1b-1,2 indicate cells transfected with the respective Rac1b siRNAs. ZR75 cells were transfected with 20 nM siRNA duplex (Ambion or Dharmacon) using Lipofectamine 2000 (Invitrogen). After 72 hrs, cell extracts were prepared and proteins were separated on an 18% gel (10 μg cell lysate, top; and 40 μg cell lysate, bottom) followed by transfer to PVDF membrane. The blots were probed with antibodies to Rac1 and α-tubulin. Results shown are representative of 6 experiments.

We have started to examine the role of Rac1b in a number of functions that are also regulated by Rac1, including the formation of lamellipodia and cell proliferation. Surprisingly, although depletion of Rac1b inhibits lamellipodia in proportion to the level of inhibition of Rac1b expression (Fig. 6A), the less potent oligo (Rac1b-1) has a strong inhibitory effect on cell proliferation, whereas the more potent oligo does not significantly affect cell proliferation at all (Rac1b-2) (Fig. 6B). These preliminary data suggest that at least one of the two oligos displays off-target effects, i.e. degradation of mRNAs that show only partial sequence identity with the region targeted in Rac1b. We therefore recently ordered two additional oligos and plan to examine their depletion potential and functional effects.
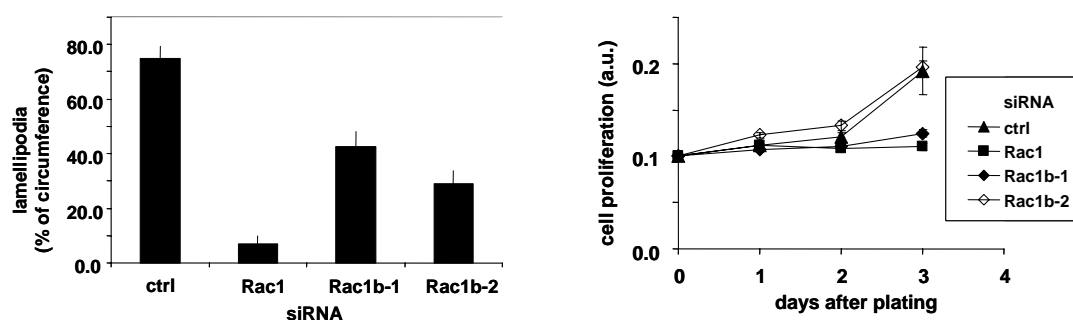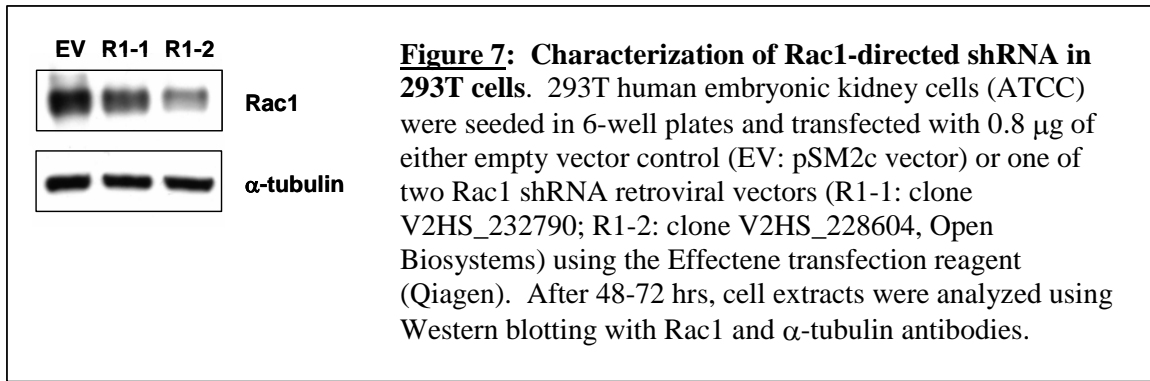
**Figure 6: Functional analysis of Rac1b in ZR75 cells**. **A**. Effect of Rac protein depletion on lamellipodia formation. Lamellipodia were quantified as the total length of lamellipodial edge divided by the circumference of the cell. At least 20 single cells were traced for each condition. Error bars denote the SEM. Transfection of ZR75 cells was performed as in Fig. 5. 72 hrs later, the cells were seeded on coverslips, serum starved overnight and then stimulated with 1 nM heregulin (R&D Systems, human heregulin1-β1 EGF domain) for 20 min. The actin cytoskeleton was visualized using Rhodamine-phalloidin and lamellipodia formation was quantified as previously described [10]. **B**. Effect of Rac protein depletion on cell proliferation. ZR75 cells were transfected as in (A). Within 24 hrs of transfection, the cells were plated at a density of $1 \times 10^4$ cells/well in 96-well plates. Cell proliferation was determined using the SRB method (see Fig. 2). Data shown are the average of 6 wells (-/+ SEM) and are representative of 3 independent experiments.

## Task 2. To determine the contribution of Rac1, Rac1b and Rac3 to CXCL12/CXCR4-mediated breast cancer metastasis.

Task 2a: Construction and production of viruses for retroviral transfection of shRNA.

We have purchased two retroviral plasmids containing independent small hairpin RNAs (shRNA) targeting Rac1 from Open Biosystems. We first tested the knock-down efficiency of these plasmids using transient transfection in 293T cells, which display a very high transfection efficiency. Only one of the two shRNAs yielded significant reduction in Rac1 protein levels (Fig. 7). We therefore are in the process of generating additional shRNA plasmids based on the Rac1-directed siRNA sequences that we have shown to be very efficient [10].

In the mean time, to test the efficiency of the commercial plasmids using retroviral transduction, we attempted to produce retroviral particles in HEK293-based (LinX) or NIH-3T3-based (PA317) packaging cells. Unfortunately, the plasmids did not yield any retroviral particles, although a control plasmid (pBABEpuro) produced the expected number of particles. Thus, as an alternative strategy, once we have obtained a more efficient shRNA plasmid, we will use transient transfection of this plasmid in T231 cells, and select pools of knock-down cells. In the event that this procedure would not yield

EV  R1-1  R1-2

Rac1

α-tubulin

**Figure 7:  Characterization of Rac1-directed shRNA in 293T cells**. 293T human embryonic kidney cells (ATCC) were seeded in 6-well plates and transfected with 0.8 μg of either empty vector control (EV: pSM2c vector) or one of two Rac1 shRNA retroviral vectors (R1-1: clone V2HS_232790; R1-2: clone V2HS_228604, Open Biosystems) using the Effectene transfection reagent (Qiagen).  After 48-72 hrs, cell extracts were analyzed using Western blotting with Rac1 and α-tubulin antibodies.

sufficient inhibition of Rac1 expression, we will try retroviral vectors that use a different promoter, such as CMV oe EF-1α.

KEY RESEARCH ACCOMPLISHMENTS

CXCL12 stimulates Rac1 activity in MDA-MB-231 breast carcinoma cells. CXCL12 also stimulates the proliferation of these cells.

REPORTABLE OUTCOMES
**None**

CONCLUSION

Research carried out during the first year of funding has been hampered by a large number of technical difficulties, including the difficulty in maintaining significant CXCR4 expression levels in breast carcinoma cells *in vitro*, problems with the generation of retroviral particles and unexpected off-target effects of Rac1b-targeting oligos. These obstacles have significantly slowed us down in completing the respective tasks that we set out to accomplish. We are currently proceeding as planned however.

Nevertheless, we were able to show that CXCL12 stimulates Rac1 activity in MDA-MB-231 breast carcinoma cells, suggesting indeed that Rac1 is likely to play a role in CXCL12-mediated functions in these cells, including cell proliferation and cytoskeletal organization. Based on these first results, we feel confident that we will be able to complete Tasks 1d and 1e (identification of candidate CXCL12-stimulated signaling elements and their role in CXCL12-stimulated proliferation and survival of breast carcinoma cells) largely within the proposed time frame. We also hope that we will be able to obtain reliable siRNA oligos to accomplish Task 1c (determination of the contribution of Rac1b to CXCL12-stimulated functions, albeit with a delay).

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APPENDICES
**none**